EXHIBIT

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BRIEF COMMUNICATION

Expression of ΔF508 CFTR in normal mouse lung after site-specific modification of CFTR sequences by SFHR

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The development of gene targeting strategies for specific modification of genomic DNA in human somatic cells has provided a potential gene therapy for the treatment of inherited diseases. One approach, small fragment homologous replacement (SFHR), directly targets and modifies specific genomic sequences with small fragments of exogenous DNA (400–800 bp) that are homologous to genomic sequences except for the desired modification. This approach has been effective for the in vitro modification of exon 10 in the cystic fibrosis transmembrane conductance regulator (CFTR) gene in human airway epithelial cells. As another step in the development of SFHR for gene therapy, studies were carried out to target and modify specific genomic sequences in exon 10 of the mouse CFTR (mCFTR) in vivo. Small DNA fragments (783 bp), homologous to mCFTR except for a 3-bp deletion (ΔF508) and a silent mutation which introduces a unique restriction site (KpnI), were instilled into the lungs of normal mice using four different DNA vehicles (AVE, LipofectAMINE, DDAB, SuperFect). Successful modification was determined by PCR amplification of DNA or mRNA-derived cDNA followed by KpnI digestion. The results of these studies showed that SFHR can be used as a gene therapy to introduce specific modifications into the cells of clinically affected organs and that the cells will express the new sequence. Gene Therapy (2001) 8, 961–965.

Keywords: *gene targeting; gene therapy; cystic fibrosis; DNA delivery vehicles*

Currently, there are two primary gene therapy approaches for the treatment of inherited disorders. One approach involves the introduction of a cDNA version of a normal gene via viral or plasmid vector into cells, or 'gene complementation'.[1] The other strategy involves site-specific modification of mutant DNA sequences by 'gene targeting'.[2,3] Gene targeting has several advantages over gene complementation, including long-term and tissue-specific expression of the functional gene, no introduction of foreign sequences and no immune response. Several gene-targeting approaches have been developed for correction of mutations associated with genetic disease. These include RNA/DNA chimeric oligos,[4–6] triplex forming oligonucleotides,[9,10] and small fragment homologous replacement (SFHR).[2,11–13]

Gene targeting by SFHR employs small DNA fragments (400–800 bp) for sequence modification to add, delete, or exchange specific base pairs. The exogenous, or small DNA, fragments are essentially homologous to the targeted endogenous DNA sequences except for the particular base pairs (bp) that encode for the desired modification. To date, SFHR has been used to modify both extra-chromosomal or episomal,[14,15] and genomic DNA sequences in human transformed and primary cells *in vitro*.[12,13] Site-specific deletion of episomal DNA sequences has been shown by the targeted correction of a defective selectable marker gene, the Zeocin resistance gene, following transient co-transfection of a plasmid containing the defective gene and small DNA fragments into transformed human epithelial cells from cystic fibrosis (CF) patients.[14,15] Site-specific addition of genomic DNA sequences has been demonstrated by the correction of a 3-bp deletion in the CFTR (CF transmembrane conductance regulator) gene of transformed and primary CF (ΔF508 genotype) airway epithelial cells that resulted in a normal, Cl^- ion transport phenotype.[12,13] In these experiments, a specific base pair modification (silent mutation) was concomitantly introduced by SFHR to create a novel restriction enzyme cleavage site within CFTR as a tag.

The efficacy of SFHR as a gene therapy has yet to be tested *in vivo*. One difficulty in going from *in vitro* to *in*

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vivo experiments is that the conditions relevant to transfer (the delivery vehicle, the target, and the route of delivery) are different. For the experiments presented here, the transfer conditions were established as follows. Given the already overwhelming body of literature on different compounds for the delivery of DNA *in vivo*,[16,17] the delivery vehicles that were chosen have either been previously used *in vitro* or have been tested for *in vivo* delivery into the airways with reporter genes. The decision for target choice was based on the fact that the primary cause of death and morbidity in CF is due to lung infections; therefore, the development of an SFHR-based gene therapy for CF would include delivery into the lung.[18] Studies were performed in the lungs of normal mice as a proof of principle. Finally, the route of delivery was intratracheal instillation thereby ensuring direct delivery of replacement fragment into the target organ, ie the airways of the lung,[19,20] as opposed to aerosol[21] or systemic delivery.[22]

The potential of SFHR as an *in vivo* gene therapy was assayed using small DNA fragments (783 bp) that were designed to introduce a site-specific 3-bp deletion in exon 10 of the endogenous mouse CFTR (mCFTR) sequence in lung cells of normal mice thereby creating a ΔF508 genotype. As an additional tag, the small DNA fragments contained a silent mutation that introduced a unique *Kpn*I restriction enzyme cleavage site into the targeted regions in the mCFTR (Figure 1). The fragments were complexed with one of four artificial DNA delivery vehicles: condensing agent and phospholipid (artificial viral envelope – AVE),[17] cationic lipid (LipofectAMINE; Life Technologies) or dimethyl-dioctadecyl-ammonium-bromide (DDAB),[23] or cationic polyamidoamine (starburst dendrimer, SuperFect, Qiagen)[24] and introduced into the lungs of normal mice via intratracheal instillation. Successful site-specific deletion of 3-bp in the mCFTR gene was determined by PCR amplification of DNA or mRNA-derived cDNA followed by *Kpn*I restriction digest. Allele-specific primers, mCF3-N and mCF3-ΔF, were used to distinguish between normal and ΔF508 sequences (Figure 1 and Table 1).

Site-specific modification of exon 10 in normal mCFTR by SFHR resulted in the expression of ΔF508 RNA as indicated by allele-specific RT-PCR analysis of RNA from the lungs of mice 3 days after intratracheal instillation of 4 μg of fragment via delivery vehicles AVE (Figure 2), LipofectAMINE (Figure 3) or DDAB (Figure 4a). In addition, PCR amplicons could be cut by *Kpn*I thus indicating that in addition to the deletion of 3 bp, the single base pair that gives rise to this unique restriction site, was modified (Figure 2 and Figure 4b). Overall, six of 13 (46%) mice tested at this dose (4 μg) were positive for the modification. Successful modification was most reproducible at this dose with the DNA vehicle AVE (three of three mice, or 100%) followed by LipofectAMINE (two of three mice, or 66%) then by DDAB (one of two mice, or 50%). Modification was least reproducible

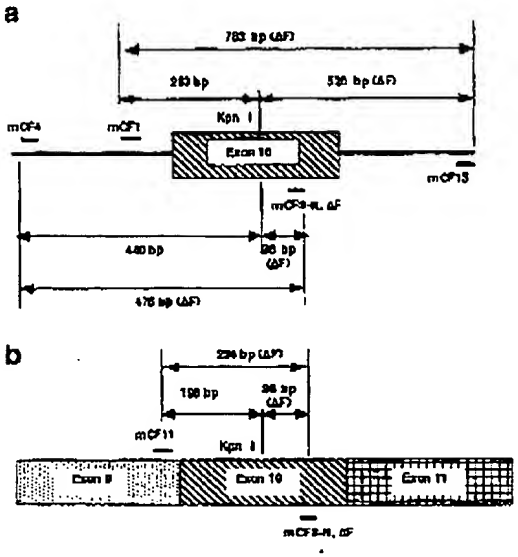

Figure 1 Schematic diagram of the PCR amplification strategy used to analyze successful SFHR of mouse CFTR. The small DNA fragments were first denatured into single-strands (ss) by boiling for 10 min and then placed on ice. Fragment (4 μg or 20 μg) was then mixed with one of four artificial DNA delivery vehicles: condensing agent and phospholipid (artificial viral envelope – AVE),[17] cationic lipid (LipofectAMINE; LifeTechnologies, Carlsbad, CA, USA) or dimethyl-dioctadecyl-ammonium-bromide (DDAB),[45] or cationic polyamidoamine (starburst dendrimer, SuperFect, Qiagen, Valencia, CA, USA)[24] and introduced into the lungs of normal mice via intratracheal instillation. Before instillation, mice (CD1, female, 5–6 wwks, 20–25 g) were anesthetized by inhalation of methoxyflurane inhalation.[32] The DNA/vehicle complex (~100 μl final volume) was instilled into the mouse lung via the trachea using a Hamilton syringe with a blunt, curved end. Control mice were instilled with isotonic solution. Afterwards, the mice were exposed to oxygen until they recovered. They were fed a normal diet and were monitored twice daily for any signs of stress until they were killed (3–7 days after instillation). The tissues (lung, trachea, heart and liver) were harvested, fast frozen in liquid nitrogen and then homogenized with a glass mortar and pestle in TRIzol Reagent (Life Technologies). DNA and RNA were purified according to the manufacturer's directions for subsequent analysis by PCR amplification. (a) The genomic target for these studies comprises mCFTR exon 10 (codons 465–528) and flanking intron regions. The small DNA fragment (783 bp) used to target this locus is defined by a region of homology on the mCFTR gene from primer mCF1 to mCF15. The fragments are homologous to normal mouse mCFTR sequences except that codon 508 (ttt) is deleted, ie ΔF508, and that a silent mutation has been introduced into codon 501 (act → acc) to create a unique KpnI site. The fragment is generated by PCR amplification with primers mCF1 and mCF15 using, as a template, a copy of the fragment that has been cloned into pBluescript SK⁺ and sequenced. Briefly, 2 ng of plasmid are amplified in 1 × PCR buffer, 2 mM $MgCl_2$, 250 μM dNTPs, 400 nM of each primer, 2 U Taq polymerase (Perkin-Elmer, Boston, MA, USA) in a 100 μl reaction (94°C 2 min, initial denaturation then 25 cycles of denaturation, 94°C for 30 s/annealing, 60°C for 30 s/extension, 72°C for 1 min, with a final extension at 72°C for 5 min). Allele-specific PCR analysis of DNA was performed using primer mCF4 (sense) and either anti-sense primer mCF3-N (specific for normal sequence) or mCF3-ΔF (specific for ΔF508 sequence). The allele-specific PCR conditions were the same as above, except that the annealing temperature was 59°C and that the amplification was carried out for 35 cycles. (b) Location of the primers for PCR amplification of mRNA-derived cDNA. Isolated RNA was first subjected to DNase (1 U DNase in 40 mM Tris, pH 8.0, 6 mM $MgCl_2$, 2 mM $CaCl_2$ at 37°C for 10 min), extracted with phenol/chloroform and precipitated with iso-propanol. The purified RNA was then reverse-transcribed (SuperScript; GibcoBRL, Bethesda, MD, USA) into first-strand cDNA. Non-allele specific PCR amplification was initially performed on the cDNA samples to insure that mCFTR was expressed and that the mRNA was not degraded (data not shown). Allele-specific PCR amplification was performed using primer mCF11 (sense) and either anti-sense primer mCF3-N (specific for normal sequence) or mCF3-ΔF (specific for ΔF508 sequence). The PCR conditions were the same as for the amplification of DNA.

Table 1 Primer sequences

Primer name	Primer sequence (5' → 3')	Sense or anti-sense	Allele-specific?
mCF4	CACACTCATGTAGTTAGAGCATAGG	sense	no
mCF1	GCCTAGAAAAGTCCCTGTATCATG	sense	no
mCF11	CTTGTGGGAAATCCTGTGCTGAA	sense	no
mCF3-N	ATCATAGGAAACACCAAA	anti-sense	yes
mCF3-ΔF	ATCATAGGAAACACCGAT	anti-sense	yes
mCF15	GGGGTCCTTGACATGTTTACAT	anti-sense	no

Figure 2 This study evaluated the ability of the artificial viral envelope (AVE) complex to facilitate DNA delivery for SFHR-mediated modification of endogenous mCFTR. Experimentally, three mice were transfected with a DNA/vehicle complex prepared using 12 μg DNA fragment, 36 μg of AVE, 6 μg protamine sulfate and 120 μg of a polypeptide fusogen (ATI 6011) that enhances uptake of the DNA complexes. After 3 days, RNA was isolated and subject to allele-specific RT-PCR (mCF11/mCF3-ΔF) followed by KpnI restriction digest. The resulting products were run on a 3% NuSieve gel with a ϕX174 HaeIII DNA ladder (MW). Lane C is a ΔF508 positive control; lane W is a water control; lanes 1–9 are from the three transfected mice (mouse 1, lanes 1–3; mouse 2, lanes 4–6; mouse 3, lanes 7–9); lanes 10–12 are from a control (normal) mouse that received glucose only. The tissues in each lane are as follows: trachea (lanes 1, 4, 7, 10), liver (lanes 2, 5, 8, 11) and lung (lanes C, 3, 6, 9, 12). Expression of ΔF-CFTR (234-bp band) was detected in the lung samples from all three transfected mice (lanes 3, 6, 9) as well as the ΔF508 control mouse. However, only PCR amplicons from the transfected mice were cut after KpnI restriction digest. The 198-bp band can be clearly seen in all three transfected samples whereas the 36-bp band cannot be resolved. The amplicon from mouse 2 is only partially digested. The non-specific band (~350 bp) has not been identified.

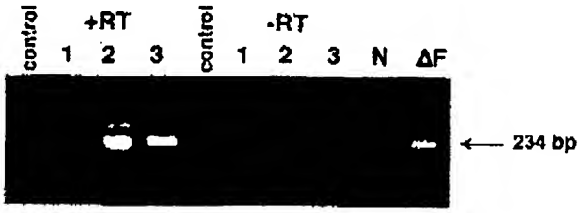

Figure 3 In this study, 4 μg of fragment was instilled into the lungs of three mice after being complexed with 7.2 μl of LipofectAMINE (1:2 negative to positive charge ratio). Tissue was harvested 3 days after transfection. Allele-specific PCR amplification (mCF11/mCF3-ΔF) was performed on mRNA-derived cDNA from lung tissue from all three mice (1–3) as well as a control (N – normal) mouse that received saline solution and a ΔF508 homozygous mouse (ΔF). The results confirm expression of exogenous ΔF508 sequences in two of the transfected mice (2 and 3) but not in mouse 1 or the control. There was no spurious amplification from samples that were not reverse-transcribed. The differences in PCR band intensity are attributed to amplification conditions and are not necessarily reflective of the amount of CFTR-RNA present in the samples.

with SuperFect (none of five mice, or 0%). The success of modification remained the same with DDAB when the amount of fragment was increased to 20 μg (one of two mice, Figure 4) and with LipofectAMINE when the amount of time after instillation was increased to 7 days (two of three mice, Figure 5).

These studies demonstrate SFHR-mediated site-specific deletion of 3 bp in endogenous mCFTR DNA and RNA in the lung cells of normal mice. These results cannot be readily attributed to a PCR artifact that entails the fragment serving as a primer in PCR amplifications. In previous experiments, false positives were not observed after allele-specific PCR amplification of genomic DNA mixed with up to the equivalent of 1000000 small DNA fragments per cell.[12] In the experiments presented here, mouse lungs were exposed to a maximum of ~57000 fragments per cell given an average lung weight of 160 mg. The actual number of fragments that were successfully delivered into cells and therefore present at the time of DNA and RNA isolation is less than 40%, resulting in at most 22800 small DNA fragments per cell.[25] In addition, a false positive due to contaminating DNA (genomic or small fragment) in the RT-PCR amplification reactions is highly unlikely because each RNA sample was treated with DNase (see Figure 1 legend).

Expression of ΔF508 mCFTR mRNA was observed in lung tissue after intratracheal instillation of the small DNA fragment with three of four DNA delivery vehicles tested using DNA:vehicle ratios that were established *in vitro*. While it is clear that there are multiple factors that can influence the success of modification by SFHR other than the delivery vehicle, the data suggests that AVE > LipofectAMINE > DDAB > SuperFect in terms of reproducibility. The finding that there was no modification after intratracheal instillation with the cationic polyamidoamine (SuperFect) may be due to the delivery system. However, we cannot rule out the possibility that those individual variations in the mice and/or the transfection conditions may play a role in the effectiveness of an individual delivery vehicle. While the results of these experiments demonstrate SFHR-mediated modification, the actual frequency of modification was not determined. The PCR amplification conditions used for analysis were designed to detect allele-specific modification of DNA and RNA sequences. To assess frequency, the PCR amplification will need to be normalized to an external control. In addition, the different rates of RNA expression and degradation between normal and ΔF508 mice need to be considered for such subsequent evaluation.

The findings presented here support observations by

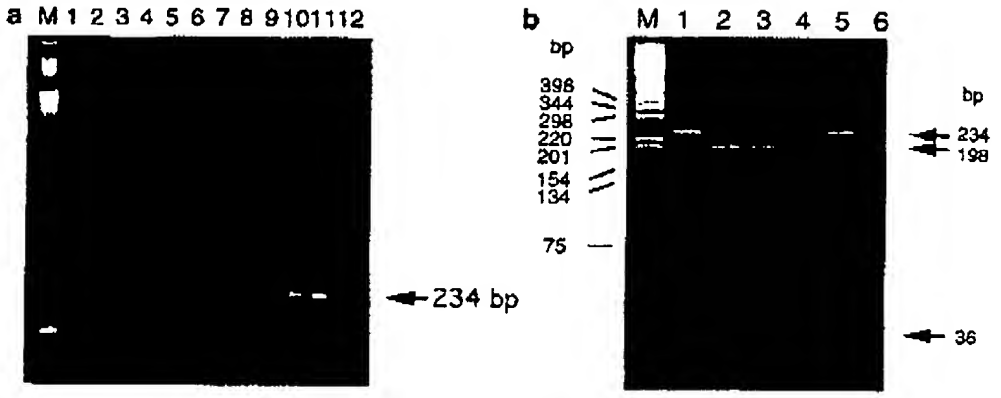

Figure 4 Expression of ΔF508 CFTR RNA after transfection of small fragments with DDAB. In this study, 80 μl of fragment (1 μg/μl) was mixed with 320 μl of DDAB and brought up to a final volume of 400 μl with 5% dextrose (first dilution). The complex was diluted again by adding 280 μl of 5% dextrose to 70 μl of the DNA/vehicle complex (second dilution). Two mice were transfected with 4 μg of fragment (100 μl of the second dilution), two mice were transfected with 20 μg of fragment (100 μl of the first dilution) and a control normal mouse received 100 μl of 5% dextrose. (a) Tissues (lung (L), trachea (T), heart (Hi) were harvested from the mice on day 4 after transfection. Allele-specific RT-PCR analysis (mCF11/mCF3-ΔF) of the RNA was performed. The PCR products from four mice are shown here: lanes 1 (L), 4 (T) and7 (H) are from one mouse transfected with 4 μg; lanes 2 (L), 5 (T) and 8 (H) are from one mouse transfected with 20 μg; lanes 3 (L), 6 (T) and 9 (H) are from the control transfected mouse; lane 10 (liver) and lane 11 (L) are from an untransfected, heterozygous (N/Δ508) mouse; lane 12 is a water control; lane M is a 100-bp DNA ladder. Allele-specific amplification of samples from the other two transfected mice did not produce a product. (b) KpnI restriction digest was performed on select mCF11/mCF3-ΔF PCR amplicons from (a). Lane 1, uncut PCR product (234 bp); lane 2, one mouse transfected with 4 μg (lane 1, part a). lane 3, one mouse transfected with 20 μg (lane 2, part a); lane 4, water control; lane 5, ΔF508 heterozygote control mouse; lane 6, water control. Only the PCR amplicons from transfected mice were cut after KpnI restriction digest. The fact that only partial digestion is seen in Figure 2 and 4b suggests that modification of both targets is not linked.

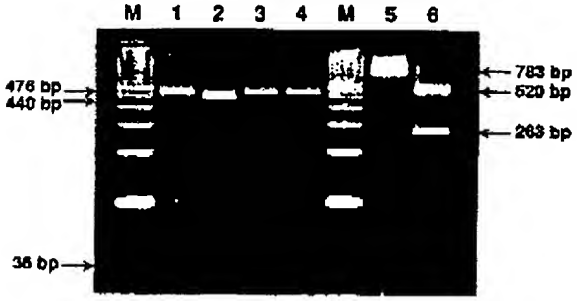

Figure 5 Successful SFHR-mediated modification of genomic mCFTR sequences. The small DNA fragment (4 μg) was complexed with Lipofect-AMINE (1:2 negative to positive charge ratio) before instillation into three mice. DNA was isolated from the lungs 3 days after transfection and allele-specific PCR was performed (mCF4/mCF3-N and mCF4/mCF3-ΔF). The results from one mouse are shown. Lane 1, mCF4/mCF3-ΔF (476 bp) uncut; lane 2, mCF4/mCF3-ΔF KpnI; lane 3, mCF4/mCF3-N (479 bp) uncut; mCF4/mCF3-N KpnI. Only the PCR product from the mCF4/mCF3-ΔF amplification is cut with KpnI. Lanes 5 and 6 show the uncut and KpnI cut small DNA fragment (783 bp). From this type of analysis, two of the three mice were shown to be positive.

others that *in vitro* conditions can be extrapolated to *in vivo* transfection, especially when the *in vitro* studies were carried out in a relevant cell type.[16,24] Given the observation that at least one mouse per experiment showed successful modification with lipid vehicles, *in vitro* experiments can be useful for defining some of the conditions for effective *in vivo* DNA transfer with lipids. The ultimate optimization of transfection efficiency will, however, require analysis *in vivo*.

On the other hand, *in vitro* conditions are not always an indication of *in vivo* success for some vehicles. In *in vitro* studies, the polyamidomine dendrimer was superior to the other delivery systems described here in airway epithelial cells.[27] However, other studies have found that the conditions for dendrimer-mediated intratracheal delivery of the DNA need to be carefully titrated.[28] As such, it is highly probable that in our case, the conditions necessary for successful *in vivo* delivery were not the same as successful *in vitro* conditions. Systemic delivery of DNA by the dendrimer complex would be an alternative route that will need to be investigated further for optimal and specific delivery.[29] Finally, it is not obvious why successful modification was more reproducible with AVE than the other vehicles, although AVE is overall negatively charged, in contrast to the net positive surface charge of the other vehicles. One possibility is that AVE may be more effective in targeting the fragments to cells that express CFTR. Alternatively, LipofectAMINE, DDAB and SuperFect complexes may not readily dissociate in the cytoplasm to facilitate nuclear delivery.[14,15]

These results indicate that SFHR has potential as a gene therapy. Previous studies have shown that cDNA can be delivered to the lung with cationic lipids for gene complementation.[20,30,31] The results presented here show that DNA fragments can also be delivered to the lung and that SFHR-mediated gene targeting is a viable route for lung/airway SFHR-mediated gene targeting. The fragments, at the doses tested here, are delivered to the appropriate cells, ie those cells expressing mCFTR mRNA. Moreover, given that ΔF508 mCFTR DNA was detected 7 days after transfection, the results suggest that SFHR effects a permanent genomic modification that would lead to RNA expression throughout the lifetime of the cell. Finally, the findings indicate that SFHR is precise in that ΔF508 mCFTR mRNA sequences were only detected within the target organs, the lung and lower air-

ways, but not in the trachea, liver, or heart (Figure 2). Thus, SFHR appears to have therapeutic potential within the airway epithelium, and with further study could be developed into a viable gene therapy for CF airways.

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